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International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C07K 7/06, A23G 1/00 | A1

(11) International Publication Number: WO 96/38472

(43) International Publication Date: 5 December 1996 (05.12.96)

(21) International Application Number: PCT/DK96/00230

(22) International Filing Date: 31 May 1996 (31.05.96)

(30) Priority Data:
0616/95 1 June 1995 (01.06.95) DK

(71) Applicant *(for all designated States except US)*: AARHUS OLIEFABRIK A/S [DK/DK]; M.P. Bruunsgade 27, P.O. Box 50, DK-8100 Aarhus C (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: RASMUSSEN, Søren [DK/DK]; Siøvej 16, DK-8370 Hadsten (DK). BACH, Mogens [DK/DK]; Provstevænget 9, DK-8464 Galten (DK).

(74) Agent: HOFMAN-BANG & BOUTARD, LEHMANN & REE A/S; Adelgade 15, DK-1304 Copenhagen K (DK).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

**Published**
*With international search report.*

(54) Title: COCOA FLAVOUR PRECURSOR PEPTIDES, DNA ENCODING THEM, PROCESSES FOR PRODUCING THE PEPTIDES, AND THEIR USE FOR GENERATING COCOA FLAVOUR

(57) Abstract

Cocoa flavour precursor peptides comprising 2-11 amino acid residues, in particular the nonapeptide Ala-Pro-Leu-Ser-Pro-Gly-Asp-Val-Phe, are isolated and characeterized from West African cocoa bens. A DNA sequence comprising the code of the peptides is synthesized, and this is inserted into replicable vectors. A recombinant host cell transformed with an expression vector containing one or more copies of the DNA sequence operably connected with control sequences which are recognized by the host cell, is cultivated to form the peptides, and these are isolated from the cultivation mixture. A cocoa flavour is produced by mixing one or more of the peptides with predominantly reducing saccharides and amino acids and roasting the mixture. The cocoa flavour may be added to food products, cosmetic products or pharmaceutical products or may be formed in situ in these.

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# Cocoa flavour precursor peptides, DNA encoding them, processes for producing the peptides, and their use for generating cocoa flavour

This invention concerns peptides which are cocoa flavour precursors, DNA encoding these peptides, vectors containing the DNA, host cells transformed therewith, and processes for producing the peptides as well as their use for generating cocoa flavour. The peptides are isolated and characterized from West African cocoa beans isolated from the cocoa tree (Theobroma cacao).

## BACKGROUND OF THE INVENTION

Cocoa beans are seeds in cocoa pods which, after harvesting, are freed from the pods and subjected to a fermentation process at or near the cultivation site, following which the greater part is exported for industrial processing. Fermented cocoa beans are roasted, giving rise to the characteristic chocolate or cocoa flavour. The subsequent grinding produces cocoa mass which is included as a main component in the chocolate production. Frequently, part of the cocoa mass is pressed, resulting in cocoa butter and cocoa powder, respectively.

The fermentation process generates heat, ethanol and in particular acetic acid, and the microorganisms as such participate only indirectly in the process. The heat activates e.g. protein, oligosaccharide and polysaccharide cleaving endogenous enzymes, which are again inactivated in the last part of the fermentation process by relatively large amounts of acetic acid. Acetic acid diffuses into the fermented beans and, in addition to direct influence on the degradation pattern and the rate, also exerts an indirect influence. The latter effect consists in

changed location of both storage protein and in lipid in the beans. The result of the fermentation is thus i.a. that some of the storage proteins are cleaved to peptides and free amino acids, and that the concentration of monomeric reducing carbohydrates is increased. The subsequent roasting subjects peptides, amino acids and reducing carbohydrates to a so-called Maillard reaction, thereby forming a cascade of flavour components. Thus, peptides serve as flavour precursors.

As early as in 1976 Mohr et al. (Mohr, W., Landschreiber, E. & Severin, T.H. (1976) Fette, Seifen, Anstrichmittel 78, 88-95) reported that amino acids and oligopeptides might be precursors of cocoa flavour. Isolate from fermented cocoa beans containing soluble carbohydrates, amino acids and peptides developed cocoa flavour during roasting. In addition, particularly Biehl and associates have contributed to the understanding of the fermentation process and proteolysis in connection with flavour generation (Biehl, B. & Passern, D (1982) J. Sci. Food Agric. 33, 1280-11290, and Biehl, B., Brunner, E., Passern, D., Quesnel, V.C. & Adamoko, D. (1985) J. Sci. Food Agric. 36, 583-598).

Cocoa and chocolate may be considered to belong to the range of food products whose flavour cannot be characterized by a single or a few flavour components. Other examples are boiled, roasted and grilled meat, baked bread and roasted coffee. The base flavour appears in all these food products as the overall impression of a balanced composition of many components.

It has unsuccessfully been attempted to imitate the said food product flavours by mixing various synthetic flavour components. A considerable problem is undoubtedly that many components contribute. By way of example, more than

500 different types of molecules have been detected in water vapour distillate from roasted cocoa beans.

Cocoa flavour mainly consists of volatile components, but the sensory experience is a combination of taste and smell sensation. Mainly two groups of chemical compounds contributing to the flavour sensation are formed during roasting. These are aldehydes which are formed by oxidative deamination of amino acids, and pyrazines formed as Maillard reaction products.

Nor have attempts at replacing the starting material been very successful.

It has been attempted to produce coffee substitutes from roasted grain or roasted chicory roots. General Foods Corporation has taken out one of the earliest patents on the production of artificial chocolate flavour by roasting various mixtures of peptides, amino acids and carbohydrates (US Patent No. 2 845 592, issued on May 20, 1958). The patent used a wide range of vegetable and animal hydrolysates, and both chemical and enzymatic hydrolysis. Hydrolysis degree of protein, concentration ratio of reactants to roasting temperature are examined. Preferred parameters are disclosed, and there are many examples of the production of cocoa flavour substitutes and use either alone or in combination with other substances. The patent represents one of the earliest literature references for cocoa flavour substitutes from other raw materials, and is drafted in very broad terms. An example of corresponding, but more recent patents in which it has been attempted to use protein hydrolysates for producing cocoa flavour, is DDR Patent No. 205 815, published on January 11, 1984, which preferably concerns enzymatically produced protein hydrolysates of gelatine and wheat gluten.

However, none of the processes referred to has been commercially successful, the reason presumably being that the flavour quality is not sufficiently good.

It is described in the International Patent Applications No. WO 91/19800 and No. WO 91/19801 from MARS UK Ltd., both published on December 26, 1991, how proteins corresponding to molecular sizes of 47 kD, 31 kD and 21 kD, respectively, were isolated from ether and acetone extracted powders of ground ripe cocoa beans. These are presumed to be subunits of the storage proteins of the cocoa bean. The polynucleotide sequences were identified, N-terminal amino acid sequences were determined, and a range of polyclonal specific antibodies for polypeptide identification was produced. A 67 kD precursor of said 47 and 31 kD proteins was identified and characterized.

Correspondingly, a 23 kD precursor of the 21 kD protein was identified and characterized. DNA encoding said 21 kD, 23 kD, 47 kD and 67 kD proteins was cloned in yeast.

The patent claims of the applications claim protection for the mentioned proteins and for fragments thereof which might conceivably be of importance for the flavour generation. Protection is also claimed for nucleic acids encoding these proteins and fragments, for their incorporation in vectors and for host cells containing these. However, it is remarkable that there is no documentation whatsoever as to which fragments might be of importance for the flavour formation, or as to how such fragments are to be produced.

## SUMMARY OF THE INVENTION

The following examples describe how a peptide having an almost optimum flavour potential has been identified in West African cocoa pods, following which the amino acid sequence was determined. An oligonucleotide was synthesized, encoding a fusion sequence between the peptide and the coding sequence of a blood factor Xa cleavage site, and the oligonucleotide was ligated into the vector pGEX-1 (Smith, D.B. & Johnson, K.S. (1988) Gene 67, 31-40), which contains a gene encoding glutathione-S-transferase, in extension of this gene. E. coli TG1 (Amersham) was transformed with the vector, and the fusion protein was expressed (Sikorski, R.S. & Hieter, P. (1989) Genetics 122, 19-27). The fusion protein was isolated by means of a glutathione "Agarose"® affinity column. Fusion protein so isolated and containing blood factor Xa cleavage site was cleaved with factor Xa and applied to the affinity column once more, whereby glutathione-S-transferase was retained on the column, and the peptide of interest was eluted. The eluate was gel-filtered on a "Superdex®75" column (Pharmacia) by means of Pharmacia FPLC equipment. The fraction containing the peptide was rechromatographed on reverse phase column, following which the identity of the peptide was confirmed by means of mass spectrometry and amino acid sequence determination by Edman degradation.

Accordingly, the invention provides a cocoa flavour precursor peptide selected from an isolated peptide with the amino acid sequence:

Lys-Ala-Pro-Leu-Ser-Pro-Gly-Asp-Val-Phe-Val

and fragments thereof containing 2-10 amino acid residues.

In particular, the peptide of the invention is selected from fragments of the peptide with the above-mentioned sequence containing 2-9 amino acid residues calculated from the alanine residue No. 2, and it is preferably a nonapeptide with the amino acid sequence:

Ala-Pro-Leu-Ser-Pro-Gly-Asp-Val-Phe.

The invention also comprises a DNA isolate comprising a DNA sequence encoding a peptide as stated above, which isolate, however, does not include the coding sequences of the 67 kD, 47 kD and 31 kD cocoa proteins.

The DNA isolate of the invention comprises in particular the DNA sequence:

$$5'\text{-AAR-GCN-CCN-}\begin{matrix}\text{TTR}\\\text{CTN}\end{matrix}\text{-}\begin{matrix}\text{TCN}\\\text{AGY}\end{matrix}\text{-CCN-GGN-GAY-GTN-TTY-GTN-3'}$$

or parts thereof of at least two codons in reading frame from the 5'-terminus, and preferably the DNA sequence:

$$5'\text{-GCN-CCN-}\begin{matrix}\text{TTR}\\\text{CTN}\end{matrix}\text{-}\begin{matrix}\text{TCN}\\\text{AGY}\end{matrix}\text{-CCN-GGN-GAY-GTN-TTY-3'}$$

or parts thereof of at least 2-8 codons from the 5'-terminus in reading frame therefrom.

A particularly useful DNA isolate of the invention, which comprises the coding sequences of a blood coagulation factor Xa cleavage site and of the above-mentioned nonapeptide as well as various restriction sites, is useful for ligation in vectors which contain a gene encoding a larger protein, so that these express a fusion protein which is easier to purify, and from which the nonapeptide can easily be released by factor Xa. This DNA isolate has the DNA sequence:

5'-GATCTTGGATCC-ATCGAGGGTCGTGCCCCATTGTCACCTGGTGACGTCTTTTAG-3'

3'-AACCTAGG-TAGCTCCCAGCACGGGGTAACAGTGGACCACTGCAGAAAATCTTAA-5'

The invention moreover comprises vectors which contain the sequence of one of the above-mentioned DNA isolates,

and in particular expression vectors which contain one or more copies of such a sequence operably linked to control sequences which are recognized by a host cell transformed with the vector.

Also recombinant host cells transformed with these vectors are comprised by the invention. Such host cells may be prokaryotes, e.g. Escherichia coli, or eukaryotes, e.g. yeast, mycelial fungi or cell lines of multi-cell organisms. Yeast, which is a well-known microorganism widely used in the food industry, must be considered particularly useful for producing cocoa flavour precursor peptides of the invention.

The invention moreover comprises various processes for producing the peptides of the invention.

Firstly, there is the process which was first used for forming and isolating the peptides from their natural sources, comprising freeing ground cocoa beans of lipids by extraction with an organic solvent and washing with acetone and an aqueous acidic buffer solution and then incubating the ground cocoa beans with an aqueous acidic buffer solution for autolysis of the proteins, following which the mass is extracted with methanol, and the extract is applied to a strong cation exchange column, from which the peptide fraction is eluted with a strong base and rapidly neutralized, and the desired peptides are isolated by chromatography.

Secondly, the one best suited for industrial production of the peptides, viz. by cultivation of a culture of a recombinant host cell, as stated above, and isolation of the resulting peptide from the cultivation mixture.

Finally, the peptides of the invention may also be produced by chemical synthesis from the individual amino acids.

The use of the peptides of the invention for producing cocoa flavour is also comprised by the invention.

Moreover, the invention comprises cocoa flavour produced by mixing of one or more peptides of the invention with predominantly reducing saccharides and amino acids and subsequent heat treatment of the mixture for 1-60 min at 100-200 °C, preferably for 5-15 min at 110-150 °C. In such a cocoa flavour, the quantitative proportion between peptide(s), saccharides and amino acids is usually

| | |
|---|---|
| peptide(s) | 30-90% by weight |
| saccharides | 10-40% by weight |
| amino acids | 0-30% by weight |

and preferably

| | |
|---|---|
| peptide(s) | 50-80% by weight |
| saccharides | 15-35% by weight |
| amino acids | 5-15% by weight |

based on the total amount of these ingredients. The saccharides in the mixture may practically consist of fructose or glucose or mixtures thereof, preferably a mixture of fructose and glucose in a weight ratio from 3:1 to 1:3.

The invention additionally comprises food products, cosmetic products and pharmaceutical products which have added thereto or contain a cocoa flavour, as stated above. Advantageously, the food products may be chocolate, confectionery, pastry or soft drinks. A particular embodiment of such products has been achieved in that during production they have been mixed with one or more peptides of the invention and, if necessary, predominantly reducing saccharides and amino acids and then sub-

jected to a heat treatment for 1-60 min at 100-200 °C, preferably for 5-15 min at 110-150 °C.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a typical reverse phase chromatogram of an extract of defatted and autolyzed cocoa beans with 70% aqueous methanol. The nonapeptide Ala-Pro-Leu-Ser-Pro-Gly-Asp-Val-Phe having a particularly high cocoa flavour potential is isolated from the peak of the chromatogram which is marked by an arrow.

Figure 2 shows a reverse phase chromatogram of said nonapeptide produced by a chemical synthesis.

Figure 3 shows a reverse phase chromatogram of a peptide material isolated from an E. coli strain which has been transformed with a plasmid containing the code of said nonapeptide sequence. The nonapeptide is detected in the peak of the chromatogram which is marked by an arrow.

Chromatography conditions of the three reverse phase chromatograms:

COLUMN: PEP-RPC HR 16/10 (Pharmacia).

MOBILE PHASE: Acetonitrile gradient, 0-20% acetonitrile of 30 min followed by 20-100% acetonitrile of 10 min in 0.1% trifluoroacetic acid (TFA).

FLOW RATE OF MOBILE PHASE: 7 ml/min.

DETECTION: UV at 214 and 280 nm.

The acetonitrile gradient is plotted in the figures. The gradient is plotted so that 0% acetonitrile is found at the base line of 214 nm detection. The base line of 280 nm detection is raised with respect to the base line of 214 nm detection on the chromatogram.

## DETAILED DESCRIPTION OF THE INVENTION

### EXAMPLE 1

**Processing of peptides**

Ripe, fresh cocoa pods from the Gold Coast, Ivory Coast were used for processing as described in this example. What was involved was a hybrid, widely distributed in the region, between two traditional cocoa tree types, Criollo and Amelonado, representing the most important African Forastero type.

After purification and disinfection in ethanol, cocoa pods were divided into two halves by a sterile scalpel. The pulp was removed, and the beans were frozen in liquid nitrogen before drying in a freeze drier.

Immediately before extraction of lipids, dried pulp residues and shell parts were removed, and the beans were crushed in a mill having a tight screen. Beans thus ground were mixed with petroleum ether and extracted in a Soxhlet device. Then the mass was filtered and the residue washed with cold acetone. Further washing was performed on an ice bath with 70% acetone admixed with 0.15% thioglycolic acid until no more colour was released. To remove the residual water, washing was completed with pure acetone, and the remaining so-called acetone residue contained proteins and protein-like compounds.

The acetone residue was then washed with cold 0.05 M citrate buffer admixed with 0.15% thioglycolic acid and 10 mM EDTA at pH 4.0. The washing procedure was repeated with a large excess of buffer. The acid washed acetone residue thus produced and containing the greater part of the proteins and protein-related compounds (including endogenic enzymes) occurring in the beans was incubated with stirring at 50 °C in 0.2 M citrate buffer admixed with 0.5% thiogycolic acid at pH 4.0.

After 24 hours the incubation was interrupted and the hydrolysate admixed with cold methanol to a final concentration of 70% by volume and an extraction volume of about 20 times the weight of defatted beans. Cold extraction (0-4 °C) was effected for 1/2 hour. Then extraction was effected once more with 70% by volume methanol, and the extracts were pooled and filtered.

To reduce any coloration of the extract, which may be ascribed to polyphenoloxidase activity in the plant tissue, adsorption was effected to polyvinyl polypyrrolidone (PVPP) at pH 2.5. Peptides and amino acids in the extract were then bound to washed and equilibrated strong cation exchanger ("Dowex® 50W"), about 2.5 ml of wet ion exchanger per gram of defatted bean. Then washing was performed in sequence with 20 and 80% 2-propanol followed by water to remove residual alcohol. The peptide fraction was liberated by basic elution (pH 10.7-11.0), and after the elution the pH value was lowered as quickly as possible to about 7 by addition of HCl. The fraction was desalted by means of cation exchanger and analyzed by reverse phase chromatography (RPC). For roasting purposes, bound peptides were eluted with ammoniumhydroxide (pH 12), which was subsequently removed as well as possible either by placement in an incubator under vacuum at 40 °C overnight or by freeze-drying.

Chromatography

The peptide fraction obtained by 24 hour autoproteolysis and isolation as described above was analyzed by means of RPC. Use was made of PEP-RPC, HR 16/10 (Pharmacia) as a stationary phase and acetonitrile gradient (0-20% acetonitrile of 30 minutes and 20-100% of 10 minutes) in 0.1% TFA (trifluoroacetic acid) as a mobile phase with a flow rate of 7 ml/min, and UV detection (214 and 280 nm). The FPLC system of Pharmacia was used, and a typical chromatogram of the said autolysate appears from figure 1.

To isolate peptides having the greatest flavour potential, the eluate was divided into three fractions, corresponding to hydrophilic and hydrophobic fractions and an intermediate fraction, respectively. It was found in roasting tests and subsequent sensory evaluation that the flavour potential was clearly best in the hydrophobic fraction and poorest in the hydrophilic fraction.

Roasting

Basically, thin layer roasting was used, as described by Mohr (Mohr, W. (1970) Fette, Seifen, Anstrichmittel 8, 695-704) comprising heat treatment at 130 $^{o}$C for 8 minutes. The peptide/protein fraction to be tested was roasted together with fructose, glucose and amino acid mixture in a weight ratio of 10:3:1:2. Samples of 20 mg were roasted (in some cases smaller amounts had to be roasted), and to ensure good contact the samples were wetted and dried in vacuum at 40 $^{o}$C before roasting.

The amino acid mixture consisted of (% by weight):

| | |
|---|---|
| Alanine | 10 |
| Arginine | 7 |
| Aspargine | 3 |
| Aspartic acid | 3 |
| Glutamine | 3 |
| Glutamic acid | 3 |
| Glycine | 2 |
| Histidine | 2 |
| Isoleucin | 5 |
| Leucin | 16 |
| Lysine | 7 |
| Methionine | 1 |
| Phenylalanine | 14 |
| Serine | 5 |
| Threonine | 4 |
| Thyroxin | 8 |
| Valine | 7 |
| | 100 |

Variations in the amino acid and sugar composition from what is stated did not necessarily change the character of the flavour by the roasting.

**Evaluation**

An in-house sensory panel was taught to reproducibly evaluate the most essential positive as well as negative flavour characters of thin layer roasted samples. The standard used was an ethanol extract from fermented, non-roasted cocoa beans as well as cocoa powder.

A result of initial studies was that the pH of the eluate should be above 8 for a good flavour development to be achieved at all, and the pH should preferably be in the range of 8 to 10. It was held that the lower pH limit was about 6, below which no good flavour development could be

obtained, even with eluates of flavour precursor peptides. It was surprisingly found that basic elution of peptides by means of ammonia water had a quite special flavour enhancing effect.

It could be demonstrated that the most important peptide eluted with about 50% acetonitrile (indicated by an arrow in figure 1) was very pure and had an excellent flavour potential. It was later found by mass spectrophotometric analysis and amino acid analysis that the peptide consisted of nine amino acids with the sequence Ala-Pro-Leu-Ser-Pro-Gly-Asp-Val-Phe. A peptide having the same amino acid sequence was produced by means of chemical synthesis methods, and this peptide had a corresponding elution profile by RPC (figure 2). The preferred approach of using the endogenic enzymes of the beans to generate flavour precursor peptides, rather than using normally fermented beans, is due to the circumstance that the polyphenol oxidase activity in cocoa beans is rather high, so that coloration and phenol derivatization of i.a. peptides and proteins are pronounced in case of long process periods with access of oxygen.

To illustrate results of a roasting test which included RPC fractionated autolysate after 24 hours' incubation of cocoa beans, the following most important characteristics are given. About 290 mg of protein/peptide were applied to the FPLC column, and eluate was collected in 40 fractions, the last two of which did not contain peptide.

| Fraction No. | Cocoa flavour | Off flavour sensation |
|---|---|---|
| 6-7 | ++ | + |
| 8 | + | + |
| 9-10 | ++ | + |
| 11 | ++ | + |
| 12-13 | (+)++ | |
| 14-16 | ++ | |
| 18-19 | + | ++ |
| 20 | + | + |
| 21-22 | + | + |
| 23 | ++ | + |
| 24 | (+)+ | (+)+ |
| 25 | + | ++ |
| 26 | + | (+) |
| 27 | + | ++ |
| 28 | (+) | + |
| 29 | + | |
| 30 | (+) | |
| 31 | + | |
| 32 | (+) | |
| 33 | (+) | (+)+ |
| 36 | ++ | |
| 37 | (+)+ | (+) |
| 38 | +++ | |

Fraction 38 was held to have a great flavour potential and was found to contain the above-mentioned nonapetide.

**Molecular cloning**

With a view to molecular cloning in E. coli of a nucleotide sequence corresponding to the identified nonapeptide, a relevant oligonucleotide having the following structure was synthesized:

BglII site - BamHI site - code of Xa recognition sequence - code of nonapeptide - stop codon - EcoRI site

as well as the complementary sequence.

The two strands were purified by polyacrylamide gel electrophoresis (PAGE) on 6% acrylamide gels containing urea (Sambrook, J., Fritsch, E.F., and Maniatis, T., (1989) In: Molecular Cloning - A Laboratory Manual. 11.23-11.28. 2nd ed., Cold Spring Harbor Lab. Press). Purified oligonucleotide strands were annealed to form the following double strand:

5'-GATCTTGGATCC-ATCGAGGGTCGTGCCCCATTGTCACCTGGTGACGTCTTTTAG-3'
3'-AACCTAGG-TAGCTCCCAGCACGGGGTAACAGTGGACCACTGCAGAAAATCTTAA-5'

Seen from the 5'-end, the first five nucleotides constitute the greater part of the BglII restriction site, which is AGATCT. The subsequent T is inserted to provide a correct reading frame. The two subsequent triplets, GGA and TCC, encode Gly and Ser, respectively, and constitute a BamHI restriction site which is inserted as a marker with a view to optional later PCR reaction. The next four triplets, ATC-GAG-GGT-CGT, encode Ile-Glu-Gly-Arg which is the recognition sequence of blood coagulation factor Xa, a very specific proteolytic enzyme which cleaves on the carboxyl side of arginine, so that the nonapeptide starts with the correct N-terminus, alanine. The triplets No. 4 and No. 3 from the 3'-end of this synthetic oligonucleotide, which encode Asp-Val, are selected from the genetic code so as to form a BsaHI restriction site, GACGTC. The last triplet on this strand, TAG, is a stop codon. The TTAA sequence at the 5'-end on the other strand constitutes four of the six nucleotides of the EcoRI restriction site.

EcoRI and BglII restriction was used for ligation into the pGEX-1 vector (Smith, D.B. & Johnson, K.S. (1988) Gene, 31-40), which contains a gene encoding glutathione-S-transferase localized so that expression in transformed bacteria causes synthesization of a fusion product between this protein and the nonapeptide, which i.a. facilitates purification and control of the expression product.

The ligated plasmid was used for transformation of E. coli strain TG1 supplied by Amersham (Hanahan, D. (1983), J. Mol. Biol. 166, 557-580). The above-mentioned BsaHI restriction site was introduced into the synthetic oligonucleotide to enable control of recombinant plasmid preparations by restriction mapping (Sambrook, J. et al. (1989), Molecular Cloning). The correct recombinant plasmids were sequenced by the dideoxy method (Sanger, F., Nicklen, S., and Coulson, A.R., (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467; Sambrook, J. et al. (1989) Molecular Cloning).

A selected strain of Escherichia coli containing the correct recombinant plasmid has been deposited under the conditions of the Budapest Treaty in Centraalbureau voor Schimmelcultures, Oosterstraat 1, P.O. Box 273, NL-3740 AG Baarn, Holland, with the Accession Number CBS 552.94.

Transformed E. coli was cultivated in shaking bottles to $A_{600}$ of 0.7-1.0 at 28 $^{o}$C, following which IPTG was added to a concentration of 0.1 mM for induction of the tac promoter. The cultures were cultivated for another 3-5 hours and then harvested.

Pelleted E. coli was resuspended in lyse buffer (50 mM Tris HCl, pH 8.0, 0.2 mg/ml of lysozyme, 1 mM EDTA) about 1:1 (weight/vol.) The suspension was incubated for 5 min-

utes at room temperature, and 0.04 (w/v) of 2% deoxycholate and 100 units/ml of "Benzonase" were added. This suspension was kept on ice for 30 minutes, and then cell residues were centrifuged off.

The supernatant was applied to a glutathione "Agarose"® affinity column equilibrated in 50 mM Tris HCl, pH 8.0 at 8 °C. The column was washed with a buffer, and the fusion protein was eluted with a buffer admixed with 5 mM reduced glutathione, dialyzed and analyzed by SDS polyacrylamide gel electrophoresis on an 18% polyacrylamide gel. The protein concentration was determined by means of the Bradford method. The yield of fusion protein was determined to be about 12 mg per g of E. coli cells (wet weight).

Factor Xa cleavage (Nagai, K. & Thøgersen, H.C. (1984) Nature 309, 810-812) was performed as described by Knudsen et al. (Knudsen, C.R., Clark, B.F.C., Degn, B., and Wiborg, O., (1992) Biochem. Int. 28, 352-362) with a few modifications. The weight ratio of protease to substrate was constantly kept at 1:200. After cleavage, affinity chromatography was again performed on the glutathione "Agarose"® affinity column, and pure nonapeptide was collected from the eluate.

Using laser mass spectrometry, the mass of the fusion protein and of the glutathione-S-transferase part of cleaved fusion protein, was determined to 27 311 and 26 409, respectively, which, in view of the uncertainty of the method, corresponds to a difference that might be ascribed to the nonapeptide.

FPLC analyses showed that a gel filtration ("Superdex® 75") was necessary to remove various contaminants from the nonapeptide. Then the same elution profile was re-

vealed under RPC (figure 3) as for nonapeptide isolated from cocoa beans and for nonapeptide produced by chemical synthesis. Plasma desorption mass spectrometry verified the identity of the microbially synthesized peptide.

## EXAMPLE 2

The sequence of the identified nonapeptide may be found as the amino acid residues Nos. 457 to 465 in the amino acid sequence of 67 kD cocoa seed storage protein precursor derived from the cDNA sequence (International Patent Application No. WO 91/19801) and in the amino acid sequence of cocoa seed vicilin derived from the gene sequence (McHenry, L. & Fritz, P.J. (1992), Plant Mol. Biol. 18, 1173-1176).

The nonapeptide isolated from cocoa beans is generated by endogenic enzyme activity and thus represents naturally produced peptides. The cleavage pattern reflects the endogenic enzyme activities under the given physical circumstances, and, of course, it is conceivable that slightly changed conditions might give rise to new peptides that might have a unique flavour potential. Therefore, the present study comprised studying the flavour potential of the nonapeptide extended by the next N-terminal amino acid, lysine, and the next C-terminal amino acid, valine, occurring in the cocoa storage protein. In addition, a plurality of minor peptides was studied, whose identities are set forth below. The peptides were synthesized by chemical methods and then purified by HPLC prior to tests in roasting experiments.

Nomenclature/peptide identity

| | |
|---|---|
| Ala-2: | Ala-Pro |
| Ala-3: | Ala-Pro-Leu |
| Ala-6: | Ala-Pro-Leu-Ser-Pro-Gly |
| Ala-7: | Ala-Pro-Leu-Ser-Pro-Gly-Asp |
| Ala-8: | Ala-Pro-Leu-Ser-Pro-Gly-Asp-Val |
| Ala-9: | Ala-Pro-Leu-Ser-Pro-Gly-Asp-Val-Phe |
| Ala-10: | Ala-Pro-Leu-Ser-Pro-Gly-Asp-Val-Phe-Val |
| Pro-7: | Pro-Leu-Ser-Pro-Gly-Asp-Val |
| Pro-8: | Pro-Leu-Ser-Pro-Gly-Asp-Val-Phe |
| Lys-10: | Lys-Ala-Pro-Leu-Ser-Pro-Gly-Asp-Val-Phe |

**Roasting - sensory evaluation**

Portions of about 20 mg each were prepared as described under "roasting" and roasted for 8 minutes at 130 $^{o}$C. The samples were evaluated shortly after the roasting. Four trained individuals participated in the sensory evaluation, and the evaluations were made independently of each other. Both positive and negative qualitative impressions were evaluated according to a scale discussed and approved beforehand, and the overall weighted evaluation is given below

| SAMPLE | SCORE | SAMPLE | SCORE |
|---|---|---|---|
| Ala-2 | 3 | | |
| Ala-3 | 4 | | |
| Ala-6 | 3 | | |
| Ala-7 | 1 | Pro-7 | 2 |
| Ala-8 | 4 | Pro-8 | 3 |
| Ala-9 | 6 | | |
| Ala-10 | 1½ | Lys-10 | 1 |

The nonapeptide was thus given a very positive evaluation when alanine was N-terminal, and thus confirmed the observations from the cocoa bean isolate. Most of the minor peptides exhibited a not inconsiderable flavour potential, and thus confirmed previous observations with fractionated cocoa bean isolate. In all experiments, the nonapeptide Ala-9 was evaluated as the clearly best one and being unique.

When lysine was N-terminal (Lys-10), the flavour potential was given a rather low evaluation. If this is compared with the evaluation of Ala-10 as well as visual observations during and after the roasting experiments, it is strongly indicated that the solubility/miscibility becomes problematic with this and greater chain lengths.

Off-flavours of a varying nature and intensity were evaluated in many samples, apart from the very best ones. It should be stressed in this connection that an unpleasant pungent odour frequently occurs when proline is the N-terminal amino acid. This may very well be ascribed to the fact that proline contains imine as a functional

group, which may be of great importance to the Maillard reaction procedure.

Further, it is worth noting that endogenic enzymes, which are responsible for the formation of the nonapeptide during incubation of cocoa beans, may have a great resemblance to trypsin and chymotrypsin and/or pepsin, respectively. This in order to be able to generate the correct terminal amino acids, alanine and phenylalanine.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Aarhus Oliefabrik A/S
(B) STREET: P.O. Box 50
(C) CITY: Aarhus C
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-8100

(ii) TITLE OF INVENTION: Cocoa flavour precursor peptides, DNA encoding them, processes for producing the peptides, and their use for generating cocoa flavour

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theobroma cacao
(B) STRAIN: Forastero

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..33
(C) IDENTIFICATION METHOD: experimental
(D) OTHER INFORMATION:/codon_start= 1
/function= "Cocoa flavour precursor"
/product= "Peptide"
/evidence= EXPERIMENTAL
/transl_except= (pos: 10 .. 12, aa: Leu)
/transl_except= (pos: 13 .. 15, aa: Ser)
/note= "The hendecapeptide and fragments thereof comprising 2-10 amino acid residues are useful cocoa flavour precursors"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AARGCNCCNN NNNNNCCNGG NGAYGTNTTY GTN 33

(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theobroma cacao
(B) STRAIN: Forastero

(ix) FEATURE:
(A) NAME/KEY: Peptide
(B) LOCATION:1..11
(D) OTHER INFORMATION:/label= Hendecapeptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Lys Ala Pro Leu Ser Pro Gly Asp Val Phe Val
1 5 10

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 27 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theobroma cacao
(B) STRAIN: Forastero

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:1..27
(C) IDENTIFICATION METHOD: experimental

(D) OTHER INFORMATION:/function= "Cocoa flavour precursor"
/product= "Peptide"
/evidence= EXPERIMENTAL
/transl_except= (pos: 7 .. 9, aa: Leu)
/transl_except= (pos: 10 .. 12, aa: Ser)
/note= "The nonapeptide is a potent cocoa flavour precursor"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GCNCCNNNNN NNCCNGGNGA YGTNTTY 27

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS:
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(v) FRAGMENT TYPE: internal

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theobroma cacao
(B) STRAIN: Forastero

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

Ala Pro Leu Ser Pro Gly Asp Val Phe
1 5

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 54 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(vi) ORIGINAL SOURCE:
(A) ORGANISM: Theobroma cacao
(B) STRAIN: Forastero

(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION:1..5
(D) OTHER INFORMATION:/note= "Larger part of BglII restriction site which is AGATCT"

(ix) FEATURE:
(A) NAME/KEY: misc_recomb
(B) LOCATION:7..12
(D) OTHER INFORMATION:/note= "A BamHI restriction site, GGATCC"

(ix) FEATURE:
(A) NAME/KEY: CDS
(B) LOCATION:13..51
(C) IDENTIFICATION METHOD: experimental
(D) OTHER INFORMATION:/product= "Fused peptide"
/evidence= EXPERIMENTAL
/note= "Fusion of recognition sequence Ile-Glu-Gly-Arg for blood coagulation factor Xa cutting 3' of Arg and cocoa flavour precursor nonapeptide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
GATCTTGGAT CC ATC GAG GGT CGT GCC CCA TTG TCA CCT GGT GAC GTC          48
              Ile Glu Gly Arg Ala Pro Leu Ser Pro Gly Asp Val
                1               5                  10

TTT TAG
Phe                                                                   54
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein
(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
Ile Glu Gly Arg Ala Pro Leu Ser Pro Gly Asp Val Phe
 1               5                  10
```

PATENT CLAIMS

1. A cocoa flavour precursor peptide selected from a peptide with the amino acid sequence (SEQ ID No. 2):

Lys-Ala-Pro-Leu-Ser-Pro-Gly-Asp-Val-Phe-Val

and fragments thereof containing 2-10 amino acid residues.

2. A peptide according to claim 1 selected from fragments of the peptide SEQ ID No. 2 containing 2-9 amino acid residues calculated from the alanine residue No. 2 in SEQ ID No. 2.

3. A peptide according to claim 1 or 2 which is a nonapeptide with the amino acid sequence (SEQ ID No. 4):

Ala-Pro-Leu-Ser-Pro-Gly-Asp-Val-Phe.

4. A DNA isolate comprising a DNA sequence encoding a peptide according to any one of claims 1-3, which isolate, however, does not include the coding sequences of the 67 kD, 47 kD and 31 kD cocoa proteins.

5. A DNA isolate according to claim 4 which comprises the DNA sequence (SEQ ID No. 1):

$$5'\text{-AAR-GCN-CCN-}\begin{matrix}\text{TTR}\\\text{CTN}\end{matrix}\text{-}\begin{matrix}\text{TCN}\\\text{AGY}\end{matrix}\text{-CCN-GGN-GAY-GTN-TTY-GTN-3'}$$

or parts thereof of at least two codons in reading frame from the 5'-terminus.

6. A DNA isolate according to claim 4 or 5 which comprises the DNA sequence (SEQ ID No. 3):

$$5'\text{-GCN-CCN-}\begin{matrix}\text{TTR}\\\text{CTN}\end{matrix}\text{-}\begin{matrix}\text{TCN}\\\text{AGY}\end{matrix}\text{-CCN-GGN-GAY-GTN-TTY-3'}$$

or parts thereof of 2-8 codons from the 5'-terminus in reading frame therefrom.

7. A DNA isolate according to any one of claims 4-6 which has the DNA sequence (SEQ ID No. 5):

5'-GATCTTGGATCC-ATCGAGGGTCGTGCCCCATTGTCACCTGGTGACGTCTTTTAG-3'
3'-AACCTAGG-TAGCTCCCAGCACGGGGTAACAGTGGACCACTGCAGAAAATCTTAA-5'.

8. A replicable vector containing the sequence of a DNA isolate according to any one of claims 4-7.

9. An expression vector containing one or more copies of a DNA isolate according to any one of claims 4-7 operably connected with control sequences which are recognized by a host cell transformed with the vector.

10. A recombinant host cell transformed with a vector according to claim 8 or 9.

11. A recombinant host cell according to claim 10 which is a yeast cell.

12. A recombinant host cell according to claim 10 which is a cell of Escherichia coli.

13. A recombinant host cell according to claim 12 which is E. coli CBS 552.94.

14. A biologically pure culture of a recombinant host cell according to any one of claims 10-13.

15. A process for producing a peptide according to any one of claims 1-3 wherein ground cocoa beans are freed of lipids by extraction with an organic solvent and washing with acetone and an aqueous acidic buffer solution and are then incubated with an aqueous acidic buffer solution for autolysis of the proteins, following which the mass is extracted with methanol, and the extract is applied to a strong cation exchange column, from which the peptide

fraction is eluted with an aqueous base and is rapidly neutralized, and the desired peptides are isolated by chromatography.

16. A process for producing a peptide according to any one of claims 1-3 by cultivation of a culture according to claim 14 and isolation of the resulting peptide from the cultivation mixture.

17. A process for producing a peptide according to any one of claims 1-3 by synthesis from the individual amino acids.

18. Use of a peptide according to any one of claims 1-3 for generating cocoa flavour.

19. A cocoa flavour produced by mixing one or more peptides according to any one of claims 1-3 with predominantly reducing saccharides and amino acids and subsequent heat treatment of the mixture for 1-60 min at 100-200 °C, preferably for 5-15 min at 110-150 °C.

20. A cocoa flavour according to claim 19 wherein the quantitative proportion between peptide(s), saccharides and amino acids is

| | |
|---|---|
| peptide(s) | 30-90% by weight |
| saccharides | 10-40% by weight |
| amino acids | 0-30% by weight |

based on the total amount of these ingredients.

21. A cocoa flavour according to claim 20 wherein the quantitative proportion between peptide(s), saccharides and amino acids is

| | |
|---|---|
| peptide(s) | 50-80% by weight |
| saccharides | 15-35% by weight |
| amino acids | 5-15% by weight |

based on the total amount of these ingredients.

22. A cocoa flavour according to any one of claims 19-21 wherein the saccharides consist of fructose or glucose or mixtures thereof.

23. A cocoa flavour according to claim 22 wherein the saccharides consist of a mixture of fructose and glucose in a weight ratio of 3:1 to 1:3.

24. A food product which has added thereto or contains a cocoa flavour according to any one of claims 19-23.

25. A food product according to claim 24 consisting of chocolate, confectionery or pastry.

26. A cosmetic product which has added thereto or contains a cocoa flavour according to any one of claims 19-23.

27. A pharmaceutical product which has added thereto or contains a cocoa flavour according to any one of claims 19-23.

28. A product according to any one of claims 24-27 to which, during production, one or more peptides according to any one of claims 1-3 and, if necessary, predominantly reducing saccharides and amino acids has been added, and which has then been subjected to a heat treatment for 1-60 min at 100-200 °C, preferably for 5-15 min at 110-150 °C.

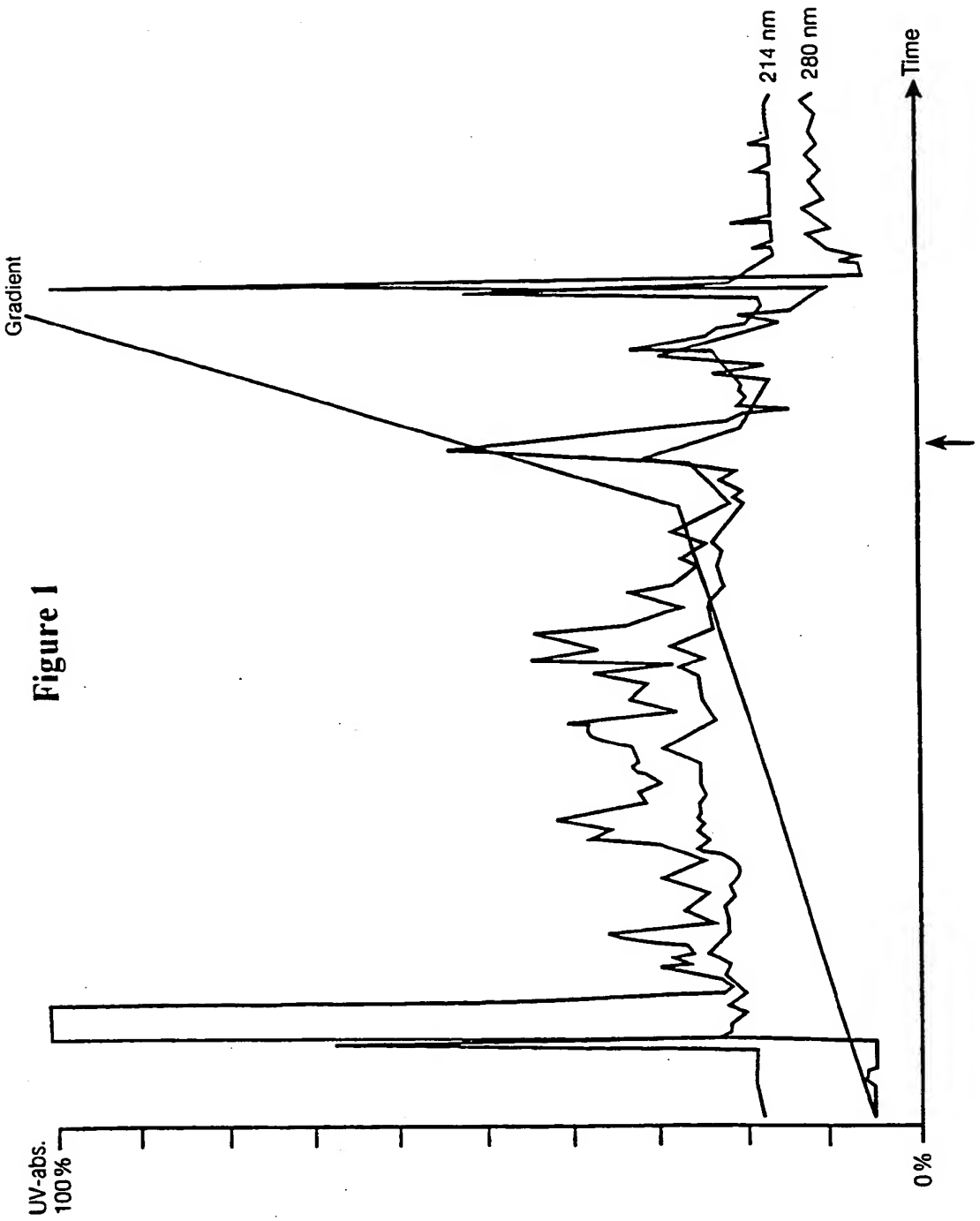


Figure 1

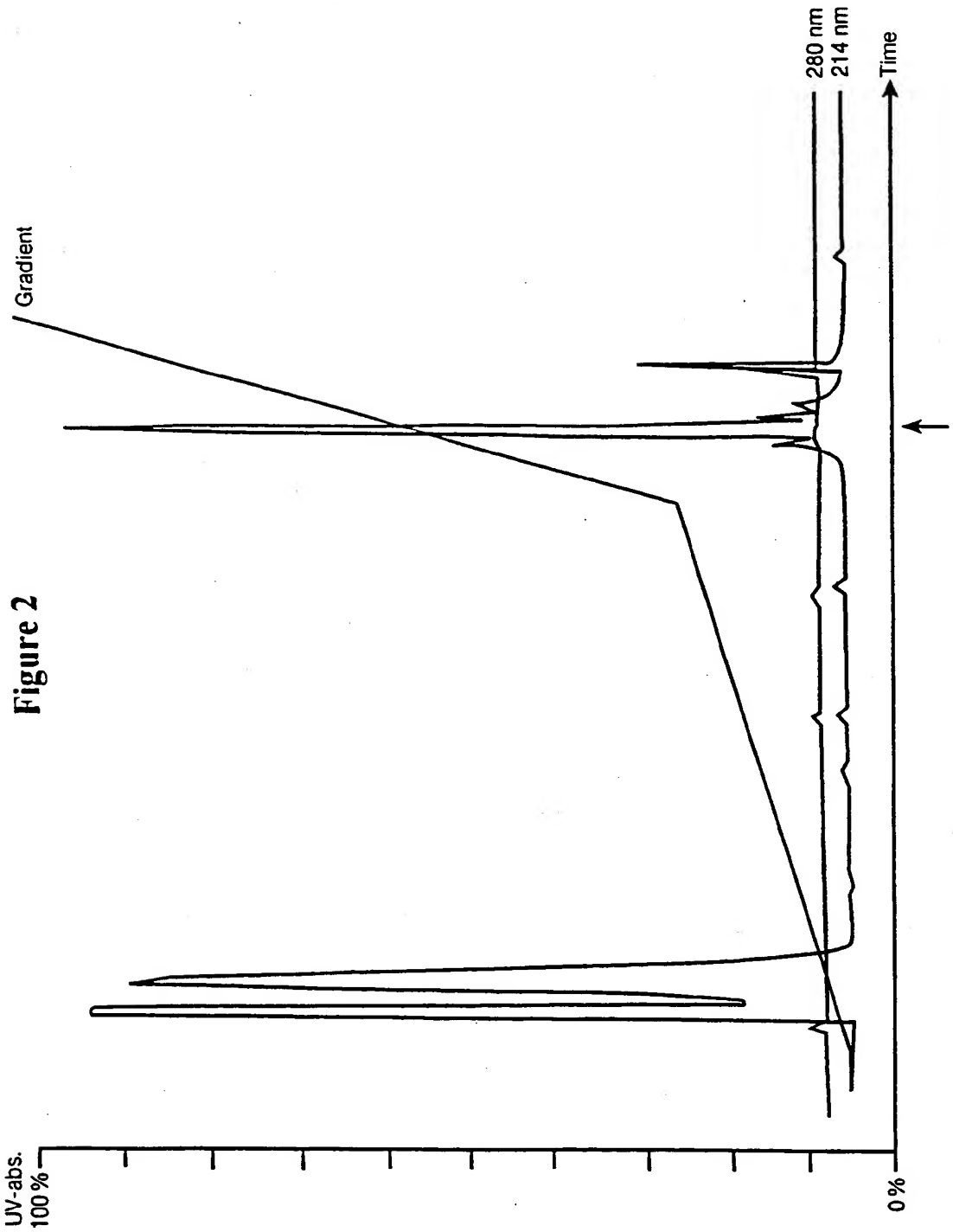


Figure 2

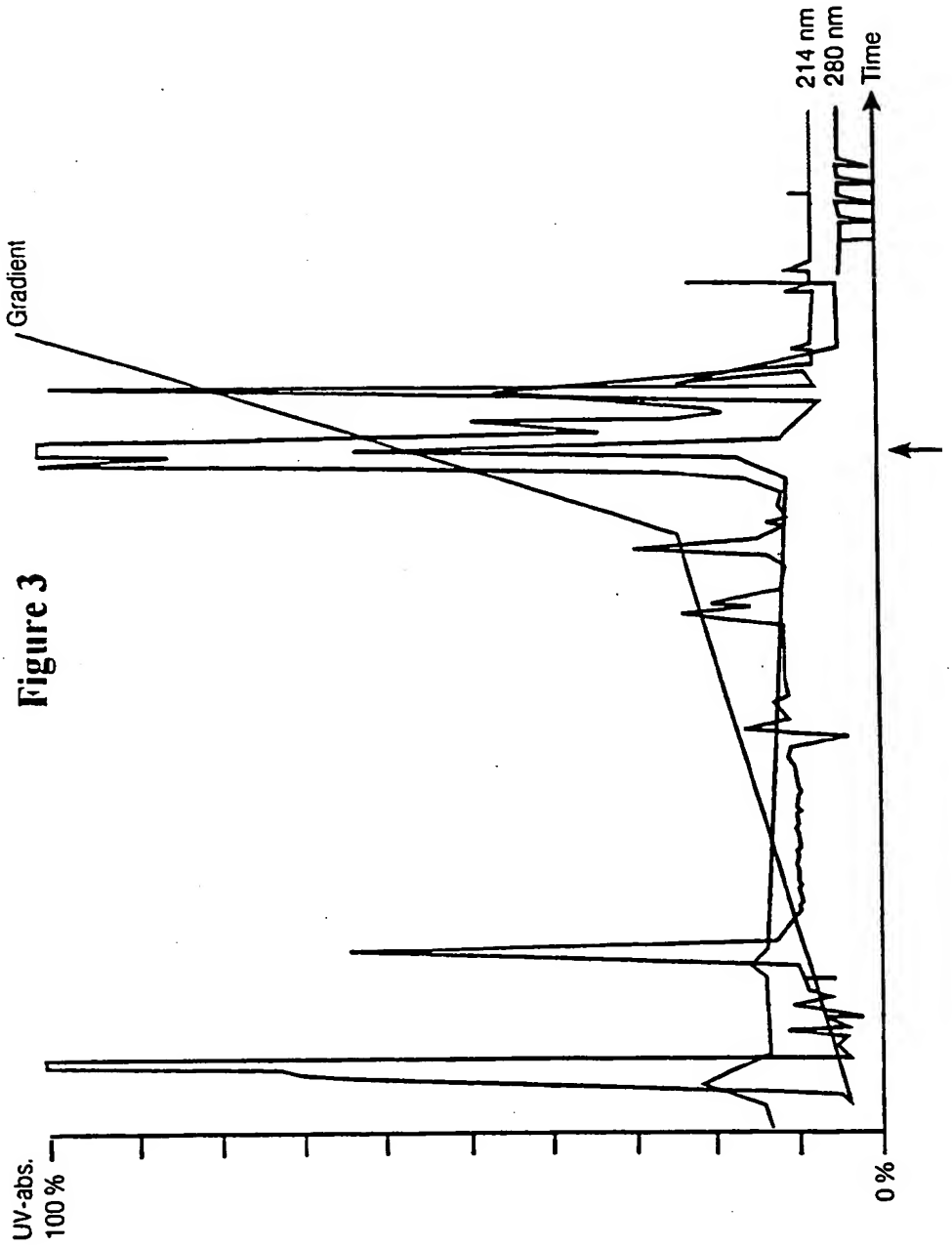


Figure 3

# INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 96/00230

A. CLASSIFICATION OF SUBJECT MATTER

IPC6: C07K 7/06, A23G 1/00
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC6: C07K, A23G

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, WPI, IFIPAT, REG, CA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| X | WO 9119801 A1 (MARS UK LIMITED), 26 December 1991 (26.12.91), figure 3 | 1-28 |
| A | Plant Molecular Biology, Volume 18, 1992, Lauren McHenry et al, "Comparison of the structure and nucleotide sequences of vicilin genes of cocoa and cotton raise questions about vicilin evolution", page 1173 - page 1176, figure 1 | 1-28 |
| A | STN International , File CAPLUS, CAPLUS accession no. 1994:433589, J. Voigt: "Proteolytic formation of cocoa flavor precursors", Prog. Flavour Precursor Stud. Proc. Int. Conf. (1993), Meeting Date 1992, 213-16. | 1-28 |

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" erlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 29 August 1996 | 02.09.1996 |
| Name and mailing address of the ISA/<br>Swedish Patent Office<br>Box 5055, S-102 42 STOCKHOLM<br>Facsimile No. +46 8 666 02 86 | Authorized officer<br>Carolina Gómez Lagerlöf<br>Telephone No. +46 8 782 25 00 |

# INTERNATIONAL SEARCH REPORT

Search request No.
PCT/DK 96/00230

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international-type search report has not been established in respect of certain claims for the following reasons:

1. ☐ Claims No.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☒ Claims No.: 1-2, 5-6
because they relate to parts of the national application that do not comply with the prescribed requirements to such an extent that no meaningful international-type search can be carried out, specifically:

The formulations "indeholdende 2-10 aminosyrerester" and "2-8 kodoner" are not clear and concise. It seems unlikely that all the fragments covered by the claims have the desired property. See Art 6.

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international-type search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international-type search report covers only those claims for which fees were paid, specifically claims No.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international-type search report is restricted to the invention first mentioned in the claims, it is covered by claims No.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

31/07/96

International application No.
PCT/DK 96/00230

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO-A1- 9119801 | 26/12/91 | AU-B- 659411 | 18/05/95 |
| | | AU-A- 7978291 | 07/01/92 |
| | | CA-A- 2084059 | 12/12/91 |
| | | EP-A- 0535053 | 07/04/93 |
| | | GB-A,B- 2260328 | 14/04/93 |
| | | HK-A- 168395 | 10/11/95 |
| | | HU-A- 65449 | 28/06/94 |
| | | HU-D- 9203913 | 00/00/00 |
| | | PL-B- 168506 | 29/02/96 |